



UNIVERSITÀ  
DEGLI STUDI  
FIRENZE

## FLORE

# Repository istituzionale dell'Università degli Studi di Firenze

### **Acetyl-L-carnitine requires phospholipase C-IP3 pathway activation to induce antinociception.**

Questa è la Versione finale referata (Post print/Accepted manuscript) della seguente pubblicazione:

*Original Citation:*

Acetyl-L-carnitine requires phospholipase C-IP3 pathway activation to induce antinociception / Nicoletta Galeotti; Alessandro Bartolini; Menotti Calvani; Raffaella Nicolai; Carla Ghelardini. - In: NEUROPHARMACOLOGY. - ISSN 0028-3908. - STAMPA. - 47:(2004), pp. 286-294. [10.1016/j.neuropharm.2004.03.016]

*Availability:*

This version is available at: 2158/326949 since: 2016-11-09T11:13:49Z

*Published version:*

DOI: 10.1016/j.neuropharm.2004.03.016

*Terms of use:*

Open Access

La pubblicazione è resa disponibile sotto le norme e i termini della licenza di deposito, secondo quanto stabilito dalla Policy per l'accesso aperto dell'Università degli Studi di Firenze (<https://www.sba.unifi.it/upload/policy-oa-2016-1.pdf>)

*Publisher copyright claim:*

(Article begins on next page)

# Acetyl-L-carnitine requires phospholipase C-IP<sub>3</sub> pathway activation to induce antinociception

Nicoletta Galeotti<sup>a,\*</sup>, Alessandro Bartolini<sup>a</sup>, Menotti Calvani<sup>a,b</sup>, Raffaella Nicolai<sup>a,b</sup>,  
Carla Ghelardini<sup>a</sup>

<sup>a</sup> Department of Pharmacology, University of Florence, Viale G. Pieraccini 6, I-50139 Florence, Italy

<sup>b</sup> Sigma-Tau Industrie Farmaceutiche Riunite S.p.A., Via Pontina km 30,400, I-00040 Pomezia, Rome, Italy

Received 26 November 2003; received in revised form 19 March 2004; accepted 24 March 2004

## Abstract

The cellular events involved in acetyl-L-carnitine (ALCAR) analgesia were investigated in the mouse hot plate test. I.c.v. pretreatment with aODNs against the  $\alpha$  subunit of G<sub>q</sub> and G<sub>11</sub> proteins prevented the analgesia induced by ALCAR (100 mg kg<sup>-1</sup> s.c. twice daily for 7 days). Administration of the phospholipase C (PLC) inhibitors U-73122 and neomycin, as well as the injection of an aODN complementary to the sequence of PLC $\beta_1$ , antagonized the increase of the pain threshold induced by ALCAR. Pretreatment with U-73343, an analogue of U-73122 inactive on PLC, did not modify ALCAR analgesic effect. In mice undergoing treatment with LiCl, which impairs phosphatidylinositol synthesis, or pretreatment with TMB-8, a blocker of Ca<sup>++</sup> release from intracellular stores, the antinociception induced by ALCAR was dose-dependently antagonized. I.c.v. treatment with heparin, an IP<sub>3</sub> receptor antagonist, prevented the increase of pain threshold induced by the investigated compound, analgesia that was restored by co-administration of D-myo-inositol. On the other hand, i.c.v. pretreatment with the selective protein kinase C (PKC) inhibitors calphostin C and chelerythrine, resulted in a dose-dependent potentiation of ALCAR antinociception. The administration of PKC activators, such as PMA and PDBu, dose-dependently prevented the ALCAR-induced increase of pain threshold. Neither aODNs nor pharmacological treatments produced any behavioral impairment of mice as revealed by the rotarod and hole board tests. These results indicate that central ALCAR analgesia in mice requires the activation of the PLC-IP<sub>3</sub> pathway. By contrast, the simultaneous activation of PKC may represent a pathway of negative modulation of ALCAR antinociception.

© 2004 Elsevier Ltd. All rights reserved.

**Keywords:** Acetyl-L-carnitine; Analgesia; Phospholipase C $\beta_1$ ; Inositol-1,4,5-trisphosphate; Protein kinase C; Cholinergic system

## 1. Introduction

Acetyl-L-carnitine ( $\gamma$ -trimethyl- $\beta$ -acetylbutyrobetaine; ALCAR), the acetyl ester of carnitine, is a small water-soluble molecule naturally present in the central nervous system involved in a wide variety of physiological functions. In the last decade, a role of ALCAR in the modulation of pain perception has been postulated. ALCAR has been demonstrated to be significantly effective in reducing neuropathic pain, a condition

characterized by spontaneous pain, allodynia and hyperalgesia caused by traumatic injury, diabetes, and viral infections. Intramuscular chronic treatment with ALCAR significantly improves the outcome of painful neuropathies or radiculopathies (Onofrj et al., 1995). A beneficial effect of ALCAR has been reported in the treatment of symptomatic diabetic neuropathy (Quatraro et al., 1995) and in the treatment of pain in distal symmetrical polyneuropathy related to HIV infection (Scarpini et al., 1997). More recently it has been reported that ALCAR induces antinociception by a central indirect cholinergic mechanism (Ghelardini et al., 2002). An up-regulation of mGlu2 receptors in ALCAR-treated animals has also been postulated (Chiechio et al., 2002). Literature data reported that

\* Corresponding author. Tel.: +39-055-4271312; fax: +39-055-4271280.

E-mail address: [nicoletta.galeotti@unifi.it](mailto:nicoletta.galeotti@unifi.it) (N. Galeotti).

the metabolic intracellular events induced by the activation of the muscarinic system are blocked not only by scopolamine, but also by the Glu2 receptor antagonist LY341495 (Johnson et al., 2000), indicating the presence of a correlation between the glutamatergic and muscarinic system, being the muscarinic system activation downstream to the mGlu2 receptors up-regulation.

Numerous reports have provided evidence for the critical involvement of the cholinergic system in pain inhibitory pathways. Several literature reports indicate that cholinergic antinociception induced both directly, through muscarinic agonists, and indirectly, by enhancing ACh extracellular levels through cholinesterase inhibitors, is mediated by  $M_1$  receptor stimulation, evidencing that  $M_1$  muscarinic receptor subtype plays an essential role in the modulation of pain perception. (Bartolini et al., 1992; Iwamoto and Marion, 1993; Ghelardini et al., 1996; Naguib and Yaksh, 1997; Ghelardini et al., 2000).

The five types of mammalian muscarinic receptors, m1 through m5, differ in primary structure as determined by molecular cloning (Caulfield, 1993). It is well established that “odd-numbered” muscarinic receptors ( $M_1$ – $M_3$ – $M_5$ ) typically couple via the  $\alpha$  subunits of the  $G_{q/11}$  family to activate phospholipase C (PLC), stimulating phosphoinositide (PI) hydrolysis (Caulfield and Birdsall, 1998). In particular, reconstitution experiments with purified m1 receptors, G protein subunits and PLC suggested that the  $\beta_1$  subtype of PLC serves as the primary effector for the m1 receptor (Felder, 1995). Receptor-mediated activation of PLC results in the generation of at least two messengers, inositol-1,4,5-triphosphate ( $IP_3$ ) and diacylglycerol (DAG). The main effect of DAG is to activate protein kinase C (PKC); the effect of  $IP_3$  is to release  $Ca^{++}$  stored in the endoplasmic reticulum. The “even-numbered” members ( $M_2$ – $M_4$ ) are preferentially coupled via  $G_i$  proteins to inhibit adenylate cyclase (Caulfield and Birdsall, 1998). Expression studies revealed that the cloned m2 and m4 receptors also stimulate PLC, although with lower efficiency than the PLC stimulation observed by m1 or m3 receptors (Ashkenazi et al., 1987).

Recently, it has been reported that the activation of the PLC- $IP_3$  pathway is essential to induce cholinergic antinociception in mice (Galeotti et al., 2003). Since it has been demonstrated that ALCAR is able to increase the pain threshold through a muscarinic mechanism, the aim of the present study was to investigate the intracellular mechanisms involved in ALCAR-mediated antinociception in mice. In particular, we examined whether the activation of PLC, and, subsequently, of the DAG- and  $IP_3$ -mediated intracellular pathways, participates in the mechanism of central antinociception following ALCAR administration.

## 2. Methods

### 2.1. Animals

Male Swiss albino mice (24–26 g) from Morini (San Polo d'Enza, Italy) were used. Twelve mice were housed per cage. The cages were placed in the experimental room 24 h before the test for acclimatization. The animals were fed a standard laboratory diet and tap water ad libitum and kept at  $22 \pm 1^\circ\text{C}$  with a 12 h light/dark cycle, light at 7 a.m. All experiments were carried out in accordance with the NIH Guide for the Care and Use of Laboratory animals. All efforts were made to minimize animal suffering, and to reduce the number of animals used.

### 2.2. Hot-plate test

The method adopted was described by O'Callaghan and Holtzman (1975). Mice were placed inside a stainless steel container, which was set thermostatically at  $52.5 \pm 0.1^\circ\text{C}$  in a precision water-bath from KW Mechanical Workshop, Siena, Italy. Reaction times (s), were measured with a stopwatch before and 15, 30, 45 and 60 min after the beginning of the test. The end-point used was the licking of the fore or hind paws. Those mice scoring less than 12 and more than 18 s in the pretest were rejected (30%). An arbitrary cut-off time of 45 s was adopted. The selection of mice by pretest values was performed before the beginning of ALCAR treatment. The pretest was also performed the day of the test in order to evaluate a possible analgesic effect induced by the treatment, but in this moment no animal was discarded.

The licking latency values reported in each figure are measured 30 min after the beginning of the test. Drugs were injected in order to obtain their maximum effect in correspondence to the 30 min after the beginning of the test.

### 2.3. Rota-rod test

The apparatus consisted of a base platform and a rotating rod with a diameter of 3 cm and a non-slippery surface. The rod was placed at a height of 15 cm from the base. The rod, 30 cm in length, was divided into five equal sections by six disks. Thus, up to five mice were tested simultaneously on the apparatus, with a rod-rotating speed of 16 rpm. The integrity of motor coordination was assessed on the basis of the number of falls from the rod in 30 s according to Vaught et al. (1985). Those mice scoring less than three and more than six falls in the pretest were rejected (20%). The performance time was measured before (pretest) and 15, 30 and 45 min after the beginning of the test. The

rota-rod experiments were performed with animals chronically treated with ALCAR.

#### 2.4. *I.c.v. injection technique*

*I.c.v.* administration was performed under ether anesthesia with isotonic saline as solvent, according to the method described by Haley and McCormick (1957). During anesthesia, mice were grasped firmly by the loose skin behind the head. A hypodermic needle (0.4 mm external diameter) attached to a 10  $\mu$ l syringe was inserted perpendicularly through the skull and no more than 2 mm into the brain of the mouse, where 5  $\mu$ l solution were then administered. We have put a restrictor on the hypodermic needle to ensure that the needle was inserted no more than 2 mm. The injection site was 1 mm to the right or left from the midpoint on a line drawn through to the anterior base of the ears. Injections were performed randomly into the right or left ventricle. To ascertain that solutions were administered exactly into the cerebral ventricle, some mice were injected with 5  $\mu$ l of diluted 1:10 India ink and their brains were examined macroscopically after sectioning. The accuracy of the injection technique was evaluated with 95% of injections being correct.

#### 2.5. *Drugs*

The following drugs were used: acetyl-L-carnitine (Sigma-Tau, Italy); TMB-8 (8-(*N,N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate) hydrochloride, heparin sodium salt (Mol. Wt: approx. 60,000), lithium chloride, (Sigma, Milan, Italy); calphostin C, chelerytrine, U-73122 (1-[6-((17 $\beta$ -3-methoxyestra-4,3,5(10)-trien-17-yl)amino)hexyl]1*H*-pyrrole-2,5-dione), U-73343 (1-[6-[[17 $\beta$ -3-methoxyestra-1,3,5(10)-trien-17-yl]amino]-hexyl]-1*H*-pyrrolidinedione), neomycin sulphate, D-myo inositol 1,4,5-trisphosphate hexasodium salt, phorbol-12,13-dibutyrate (PDBu), phorbol-12-myristate-13-acetate (PMA) (Calbiochem, Milan, Italy). Other chemicals were of the highest quality commercially available. U-73122, U-73343, calphostin C, chelerytrine, PMA, PDBu were dissolved in 0.5% DMSO whereas all other drugs were dissolved in isotonic (NaCl 0.9%) saline solution immediately before use. Drug concentrations were prepared so that the necessary dose could be administered in a volume of 5  $\mu$ l per mouse by intracerebroventricular (*i.c.v.*) injection and 10 ml kg<sup>-1</sup> by subcutaneous (*s.c.*) injection. ALCAR was administered twice daily for 7 days. The last injection of ALCAR was performed the day before the beginning of experiments and, exactly, 12 h prior to the test. Therefore, the licking latency values reported in each figure for the ALCAR-treated group represent the effect produced by a 7-day treatment with ALCAR. PMA, PDBu and Calphostin C were injected *i.c.v.* 1 h

before the test; chelerytrine was injected 90 min before the test; LiCl was administered *s.c.* 18 h before the test; U-73122, U-73343, neomycin, TMB-8, heparin, D-myo inositol were injected 15 min before behavioral tests. Doses and administration schedule were chosen on the bases of time-course and dose-response experiments previously performed in our laboratory. Furthermore, literature data confirm the selectivity and efficacy of the above-mentioned treatments at time and concentration used.

##### 2.5.1. *Antisense oligonucleotides*

Phosphodiester oligonucleotides (ODNs) protected by terminal phosphorothioate double substitution (capped ODNs) against possible exonuclease-mediated degradation were purchased from Tib-Molbiol (Genoa, Italy). The sequences are the following: anti-G<sub>q $\alpha$</sub> : 5'-C\*G\*G CTA CAC GGT CCA AGT C\*A\*T-3', corresponding to nucleotides 484–504 of the G<sub>q $\alpha$</sub>  sequence; anti-G<sub>11 $\alpha$</sub> : 5'-C\*T\*T\*G TGG CGA TGC GGT CCA C\*G\*T-3', corresponding to nucleotides 487–507 of the G<sub>11 $\alpha$</sub>  sequence; anti-PLC $\beta$ <sub>1</sub>: 5'-G\*C\*T GTC GGA CAC G\*C\*A-3', corresponding to nucleotides 49–63 of the PLC $\beta$ <sub>1</sub> gene sequence. All aODNs were previously characterized by *in vitro* and *in vivo* experiments (Sanchez-Blazquez and Garzon, 1998). We also confirmed the aODN effect on G $\alpha$  and PLC $\beta$ <sub>1</sub> protein levels by performing immunoblotting experiments. We observed a statistically significant reduction of the expression of G<sub>q $\alpha$</sub> , G<sub>11 $\alpha$</sub>  and PLC $\beta$ <sub>1</sub> after aODN treatment in comparison with mice treated with dODN (data not shown). A 21-mer fully degenerated ODN (dODN) 5'-N\*N\*N NNN NNN NNN NNN NNN N\*N\*N-3' (where N is G, or C, or A, or T) and a 15-mer fully degenerated ODN (dODN) 5'-N\*N\*N NNN NNN NNN N\*N\*N-3' (where N is G, or C, or A, or T) were used as a control respectively for the 21-mer anti-G<sub>q $\alpha$</sub>  and anti-G<sub>11 $\alpha$</sub>  and for the 15-mer anti-PLC $\beta$ <sub>1</sub>. ODNs were vehiculated intracellularly by an artificial cationic lipid (DOTAP, Sigma, Italy) to enhance both uptake and stability. aODN or dODN were preincubated at 37 °C for 30 min with 13  $\mu$ M DOTAP and supplied to mice by *i.c.v.* injection of 5  $\mu$ l solution 72, 48 and 24 h prior to the behavioral tests.

#### 2.6. *Statistical analysis*

All experimental results are given as the mean  $\pm$  S.E.M. An analysis of variance ANOVA, followed by Fisher's Protected Least Significant Difference procedure for post-hoc comparison, were used to verify significance between two means of behavioral results. Data were analyzed with the StatView software for the Macintosh (1992). *P* values of less than 0.05 were considered significant.

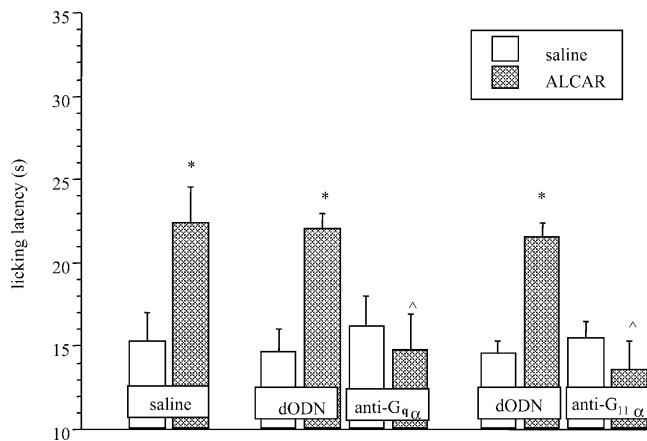


Fig. 1. Prevention by pretreatment with anti-G<sub>qα</sub> (3 nmol per mouse i.c.v.) and anti-G<sub>11α</sub> (3 nmol per mouse i.c.v.) of ALCAR (100 mg kg<sup>-1</sup> s.c. twice daily for 7 days)-induced antinociception in the mouse hot-plate test. The licking latency values were recorded 30 min after the beginning of the test. ODNs were administered 72, 48 and 24 h prior to the behavioral tests. Vertical lines represent s.e. mean. \**P* < 0.05 in comparison with saline, ^*P* < 0.05 in comparison with dODN + ALCAR-treated mice. The number of animals ranged between 15 and 18.

### 3. Results

#### 3.1. Role of G<sub>qα</sub> and G<sub>11α</sub> on ALCAR antinociception

Previous experiments performed in our laboratory showed an analgesic effect induced by ALCAR (100 mg/kg sc administered twice daily for 7 days) that persisted up to 7 days after the end of the 7-day treatment. The increase of the pain threshold induced by ALCAR disappeared 21 days after the end of the treatment (Ghelardini et al., 2002).

The effect of pretreatment with antisense ODNs (aODN) to the α subunit of G<sub>q</sub> and G<sub>11</sub> proteins on ALCAR (100 mg kg<sup>-1</sup> s.c. twice daily for 7 days) induced antinociception was evaluated in the mouse hot-plate test. Anti-G<sub>qα</sub> (3 nmol per mouse i.c.v.) and anti-G<sub>11α</sub> (3 nmol per mouse i.c.v.) antagonized the increase of the pain threshold produced by ALCAR (Fig. 1). The aODNs, when injected alone, did not modify the pain threshold in comparison with control animals. Furthermore, the i.c.v. injection of dODN (3 nmol per mouse i.c.v.), used as control, neither modified the licking latency of mice nor altered the sensitivity to analgesic drugs in comparison with saline-pretreated animals (Fig. 1).

#### 3.2. Role of PLCβ<sub>1</sub> on ALCAR antinociception

The administration of the PLC inhibitor U-73122 prevented ALCAR (100 mg kg<sup>-1</sup> s.c. twice daily for 7 days) antinociception. The dose of 0.6 μg per mouse i.c.v. was devoid of any effect whereas a complete prevention was reached at 2.5 μg per mouse i.c.v. (Fig. 2).

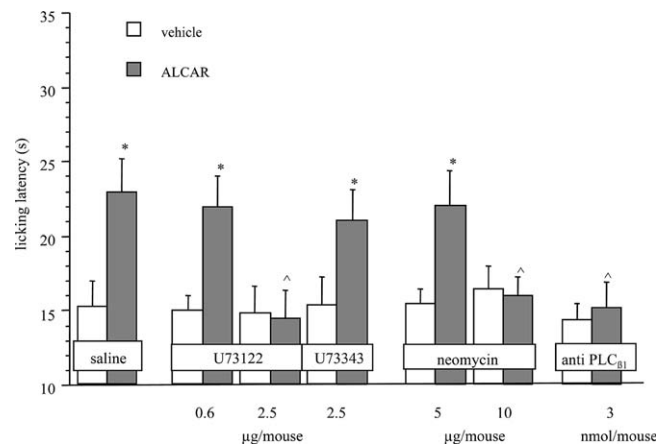


Fig. 2. Prevention by pretreatment with U-73122 (0.6–2.5 μg per mouse i.c.v.), neomycin (5–10 μg per mouse i.c.v.) and anti-PLCβ<sub>1</sub> (3 nmol per mouse i.c.v.) of ALCAR (100 mg kg<sup>-1</sup> s.c. twice daily for 7 days)-induced antinociception in the mouse hot-plate test. The licking latency values were recorded 30 min after the beginning of the test. The vehicle is: 0.5% DMSO for U-73122 and U-73343, saline for neomycin, dODN for anti-PLCβ<sub>1</sub>. ODNs were administered 72, 48 and 24 h prior to the behavioral tests. \**P* < 0.05 in comparison with control group, ^*P* < 0.05 in comparison with ALCAR-treated mice. The number of animals ranged between 14 and 16.

By contrast, U-73343 (2.5 μg per mouse i.c.v.), an analogue of U-73122 inactive on PLC, used as negative control, did not alter the increase of pain threshold produced by ALCAR (Fig. 2).

The PLC inhibitor neomycin (10 μg per mouse i.c.v.) prevented the ALCAR analgesic effect. At a lower dose (5 μg per mouse i.c.v.) neomycin was devoid of any effect (Fig. 2).

Pretreatment with an anti-PLCβ<sub>1</sub> (3 nmol per mouse i.c.v.) prevented the increase of the pain threshold induced by ALCAR (Fig. 2). The i.c.v. injection of dODN (3 nmol per mouse i.c.v.), used as control, neither modified the licking latency of mice nor altered the sensitivity to analgesic drugs in comparison with naive and saline-treated animals (data not shown).

U-73122, neomycin and anti-PLCβ<sub>1</sub>, when injected alone, produced neither a hyperalgesic nor an analgesic effect (Fig. 2).

#### 3.3. Role of IP<sub>3</sub> on ALCAR-induced antinociception

Pretreatment with LiCl, which impairs phosphatidylinositol synthesis, 18 h before the test dose-dependently reduced the antinociception induced by ALCAR (100 mg kg<sup>-1</sup> s.c. twice daily for 7 days). LiCl, at 40 mg kg<sup>-1</sup> s.c., was devoid of any effect. The dose of LiCl of 200 mg kg<sup>-1</sup> s.c. reduced ALCAR analgesic effect without reaching the statistical significance whereas the dose of 400 mg kg<sup>-1</sup> s.c. significantly prevented the increase of the pain threshold induced by the cholinesterase inhibitor. The injection of LiCl (40–400 mg kg<sup>-1</sup> s.c.) alone did not modify the licking



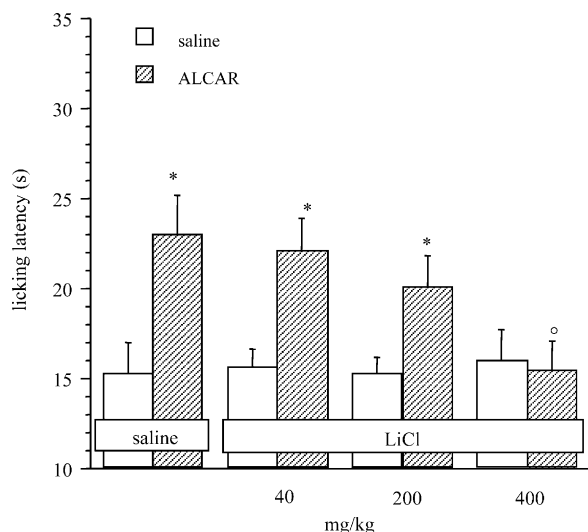


Fig. 3. Prevention by pretreatment with LiCl (40–400 mg kg<sup>-1</sup> s.c.) of ALCAR (100 mg kg<sup>-1</sup> s.c. twice daily for 7 days)-induced antinociception in the mouse hot-plate test. The licking latency values were recorded 30 min after the beginning of the test. Vertical lines represent s.e. mean. <sup>o</sup>*P* < 0.05 in comparison with saline-treated mice, \**P* < 0.05 in comparison with ALCAR-treated mice. The number of animals ranged between 13 and 15.

latency of mice in comparison with saline-treated animals (Fig. 3).

The administration of heparin (1–40 µg per mouse i.c.v.), an antagonist of IP<sub>3</sub> receptors, prevented the increase of the pain threshold induced by ALCAR (100 mg kg<sup>-1</sup> s.c. twice daily for 7 days) (Fig. 4). The dose of heparin of 1 µg per mouse i.c.v. was devoid of any effect. Co-administration of D-myo-inositol (1 µg per mouse i.c.v.) reverted the heparin-induced antagon-

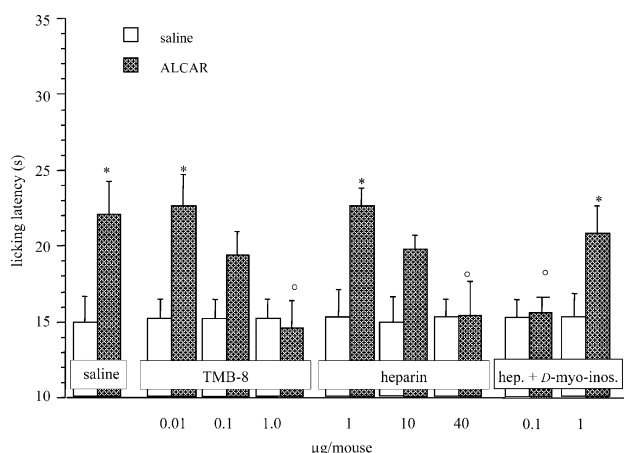


Fig. 4. Prevention by TMB-8 (0.01–1 µg per mouse i.c.v.) and heparin (1–40 µg per mouse i.c.v.) of ALCAR (100 mg kg<sup>-1</sup> s.c. twice daily for 7 days)-induced antinociception in the mouse hot-plate test and reversal of heparin antagonism by D-myo-inositol (1 µg per mouse i.c.v.). Vertical lines represent s.e. mean. \**P* < 0.05 in comparison with saline-treated mice; <sup>o</sup>*P* < 0.05 in comparison with ALCAR-treated mice. The number of animals ranged between 14 and 18.

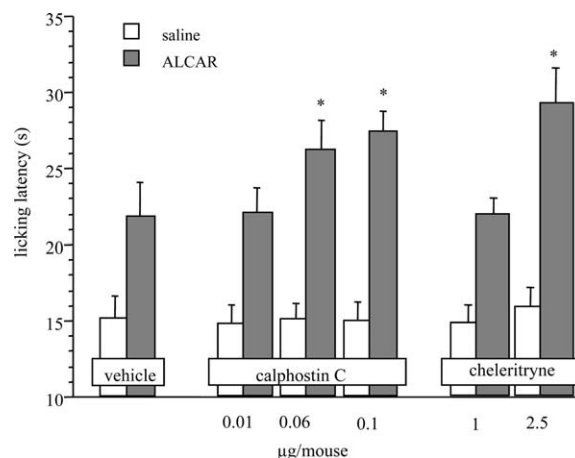


Fig. 5. Potentiation by calphostin C and chelerythrine of ALCAR (100 mg kg<sup>-1</sup> s.c. twice daily for 7 days)-induced antinociception in the mouse hot-plate test. The licking latency values were recorded 30 min after the beginning of the test. Vertical lines represent s.e. mean. \**P* < 0.05 in comparison with ALCAR-treated mice. The number of animals ranged between 12 and 16.

istic effect, whereas, at the dose of 0.1 µg per mouse i.c.v. did not modify the antagonistic effect produced by heparin (Fig. 4).

TMB-8, a blocker of Ca<sup>++</sup> release from intracellular stores, antagonized ALCAR (100 mg kg<sup>-1</sup> s.c. twice daily for 7 days) antinociception. The dose of TMB-8 of 0.01 µg per mouse i.c.v. was ineffective; the dose of 0.1 µg per mouse i.c.v. reduced the increase of the pain threshold induced by the ALCAR without reaching the statistical significance whereas the maximum antagonistic effect was reached at 1 µg per mouse i.c.v. (Fig. 4). TMB-8, when administered alone, did not modify the licking latency values in comparison with control animals (Fig. 4).

### 3.4. Role of PKC on ALCAR-induced antinociception

Pretreatment with the PKC blocker calphostin C produced a dose-dependent potentiation of ALCAR (100 mg kg<sup>-1</sup> s.c. twice daily for 7 days) antinociception in the mouse hot-plate test. The maximum effect was obtained at the dose of 0.1 µg per mouse i.c.v. The dose of 0.01 µg per mouse i.c.v. was devoid of any effect (Fig. 5). Similarly to calphostin C, chelerythrine, another PKC blocker, potentiated the antinociceptive effect of ALCAR at the dose of 2.5 µg per mouse (Fig. 5).

Calphostin C and chelerythrine potentiated the increase of the pain threshold induced by ALCAR, without modifying the mouse pain threshold when administered alone (Fig. 5).

The PKC activators PDBu (10–40 pmol per mouse i.c.v.) and PMA (1–15 pmol per mouse i.c.v.) dose-dependently antagonized the analgesia induced by

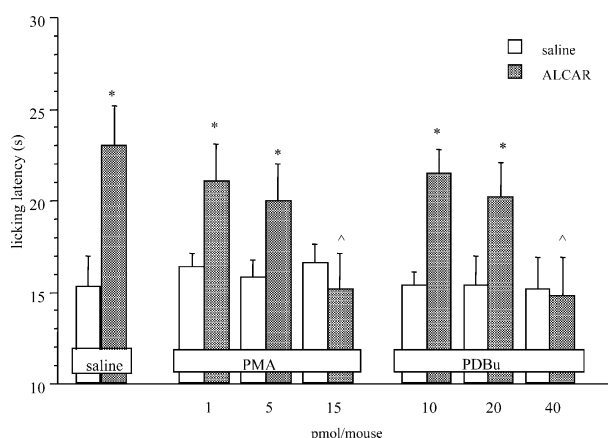


Fig. 6. Prevention by pretreatment with PDBu (10–40 pmol per mouse i.c.v.) and by PMA (1–15 pmol per mouse i.c.v.) of ALCAR (100 mg kg<sup>-1</sup> s.c. twice daily for 7 days)-induced antinociception in the mouse hot-plate test. The licking latency values were recorded 30 min after the beginning of the test. Vertical lines represent s.e. mean. \* $P < 0.05$  in comparison with saline-treated mice, ^ $P < 0.05$  in comparison with ALCAR-treated mice. The number of animals ranged between 13 and 16.

ALCAR (100 mg kg<sup>-1</sup> s.c. twice daily for 7 days) (Fig. 6). The maximum antagonistic effect of PDBu and PMA was obtained, respectively, at the dose of 40 and 15 pmol per mouse i.c.v.; at the highest effective doses the two PKC activators did not modify the animals' licking latency values in comparison with control animals (Fig. 6).

### 3.5. Effect of treatments on mouse behavior

The compounds investigated, at the highest effective doses, were tested in order to assess their effect on mouse behavior. Mice pretreated with aODNs (3.0 nmol per mouse i.c.v.), dODN (3.0 nmol per mouse i.c.v.), LiCl (400 mg kg<sup>-1</sup> s.c.), TMB-8 (1 µg per mouse i.c.v.), D-myo inositol (1 µg per mouse i.c.v.), heparin (40 µg per mouse i.c.v.), U-73122 (2.5 µg per mouse i.c.v.), U-73343 (2.5 µg per mouse i.c.v.), neomycin (10 µg per mouse i.c.v.), calphostin C (0.1 µg per mouse i.c.v.), chelerytrine (2.5 µg per mouse i.c.v.), PMA (15 pmol per mouse i.c.v.), PDBu (40 pmol per mouse i.c.v.) were evaluated for motor coordination by use of the rota-rod test, and for spontaneous motility and inspection activity by use of the hole board test.

The endurance time, evaluated before and 15, 30 and 45 min after the beginning of the rota-rod test, showed the lack of any impairment in the motor coordination of animals pretreated with aODNs in comparison with dODN group. Mice pretreated with LiCl, TMB-8, D-myo inositol, heparin, neomycin or pretreated with U-73122, U-73343, calphostin C, chelerytrine, PMA, PDBu did not show any alteration of motor coordination in comparison, respectively, with saline or

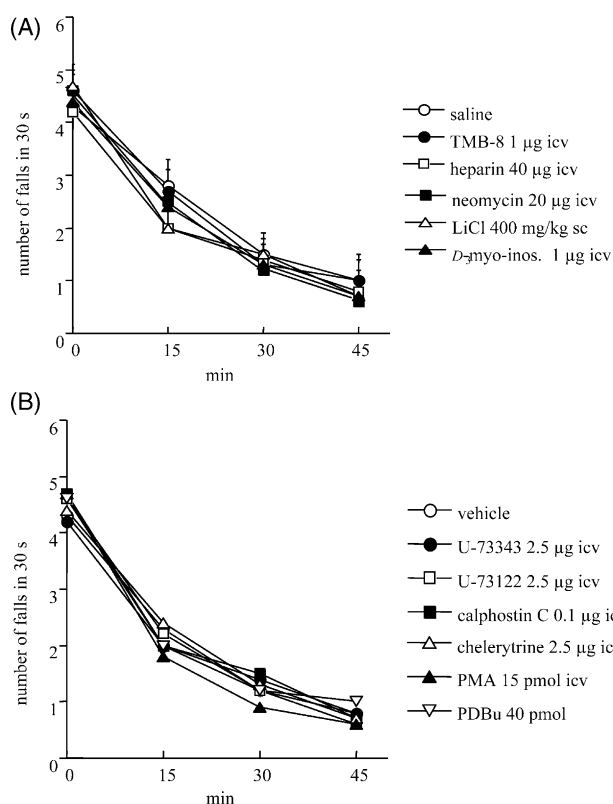


Fig. 7. Lack of effect of intracellular pharmacological modulators on motor coordination in the mouse rota-rod test in comparison with saline (panel A) or vehicle (panel B). Vertical lines represent s.e. mean. Ten animals per group were used.

vehicle group (Fig. 7). In order to further exclude any impairment of motor coordination induced by the above-mentioned treatments, we also performed the rota rota test by pretraining animals and obtaining the same results (data not shown).

Higher doses of the intracellular modulators employed were not investigated since they induced behavioral side effects such as tremors and convulsions.

## 4. Discussion

ALCAR antinociception has been reported to underlie the indirect activation of the central muscarinic system (Ghelardini et al., 2002). The present study investigated the intracellular mechanism involved in ALCAR-induced increase of pain threshold in a condition of acute thermal nociception in mice and demonstrated the importance of the activation of the PLC-IP<sub>3</sub> pathway in ALCAR analgesia.

Several PLC inhibitors are currently available. U-73122, an aminosteroid, has been found to be a potent inhibitor of aggregation of human platelets induced by a variety of agonists, and this compound has been further characterized as an inhibitor of G protein-medi-

ated PLC (Wakdo et al., 1983; Yule and Williams, 1992). Neomycin has been reported to inhibit hormone-stimulated  $IP_3$  production through the blockade of PLC (Phillippe, 1994). The administration of U-73122 dose-dependently prevented the antinociception induced by ALCAR. By contrast, U-73343, a succinimido analogue used as negative control for U-73122 being a weak inhibitor of PLC (Bleasdale et al., 1990; Smith et al., 1990), did not modify the analgesia induced by ALCAR suggesting that the prevention of ALCAR-induced analgesia by U-73122 is mediated by PLC activation. This hypothesis is also supported by the reversal of ALCAR antinociception produced by neomycin. Phosphoinositide-specific PLC represents a family of isozymes found in eukaryotes composed by  $\beta$ ,  $\gamma$  and  $\delta$  subtypes that cleaves the polar head group from inositol lipids (Rebecchi and Pentyala, 2000). Present results indicate that ALCAR activates the PLC pathway and provides evidence for the involvement of the PI-specific PLC ( $PLC\beta$ ). Among the  $PLC\beta$  subfamily, the isozyme  $PLC\beta_1$  has been reported to be selectively activated by  $M_1$  receptors (Felder, 1995), a muscarinic receptor subtype mainly involved in the induction of muscarinic analgesia (Ghelardini et al., 2000). In order to elucidate the role of this PLC subtype in ALCAR antinociception, an aODN complementary to the sequence of  $PLC\beta_1$  was employed. The inhibition of the expression of this isozyme prevented the increase of pain threshold induced by ALCAR. These results indicate PLC, and in particular the isozyme  $PLC\beta_1$ , as an important intracellular effector in the analgesic effect induced by the investigated compound.

The “odd-numbered” muscarinic receptors activate PLC via the  $\alpha$  subunit of the  $G_{q/11}$  proteins (Caulfield and Birdsall, 1998). Furthermore, activation of  $PLC\beta_1$  is achieved with the help of  $G\alpha$  subunits of the  $G_q$  proteins (Taylor et al., 1991). The administration of aODNs against the  $\alpha$  subunit of  $G_q$  and  $G_{11}$  proteins antagonized the ALCAR antinociception. These results indicate that the stimulation of the PLC-mediated intracellular pathway in ALCAR analgesia requires the receptor-mediated activation of  $G_{q/11}$  transducer proteins.

PLC isozymes hydrolyze the highly phosphorylated lipid phosphatidylinositol 4,5-bisphosphate generating two intracellular products:  $IP_3$ , a universal calcium-mobilizing second messenger, and DAG, an activator of PKC. To investigate the  $IP_3$ -mediated pathway, LiCl, an uncompetitive inhibitor of inositol monophosphatase, which regenerates inositol from inositol monophosphate, was used. The LiCl-induced inhibition depletes inositol and prevents the formation of  $IP_3$  (Kennedy et al., 1990; Phiel and Klein, 2001). Animals pretreated with LiCl showed an impaired antinociceptive response to administration of ALCAR suggesting the involvement of the PLC- $IP_3$  pathway in the

intracellular mechanism of ALCAR. The role of  $IP_3$  in ALCAR analgesia was also confirmed by the dose-dependent prevention of the increase of pain threshold induced by the above-mentioned compound by low molecular weight heparin administration. A reversal of the heparin antagonistic effect was obtained after co-administration of D-myo inositol 1,4,5-trisphosphate, further supporting the hypothesis of the activation of the PLC- $IP_3$  pathway in ALCAR analgesia. Heparin is a potent and selective  $IP_3$  receptor antagonist (Jonas et al., 1997). This compound must be injected into cells or perfused onto permeabilized cells because of its high molecular weight (12,000–13,000 Da) and lack of membrane permeability. Some evidence indicates that the low molecular weight heparin (6000 Da) used in this study is membrane permeable. Perfusion of low molecular weight heparin over a non-permeabilized cerebellar slice preparation attenuated glutamate-stimulated increases in free intracellular  $Ca^{++}$  (Jonas et al., 1997).  $IP_3$ , through the interaction with specific receptors located on the endoplasmic reticulum, causes release of  $Ca^{++}$  from intracellular stores into the cytoplasm (Mignery and Sudhof, 1990; Ferris et al., 1992). Since present results indicate the importance of  $IP_3$  production in the induction of ALCAR analgesia, we thought it worthwhile to investigate the role played by the variation of the intracellular  $Ca^{++}$  levels in the mechanism of action of the investigated compound. To this purpose, TMB-8, an agent that antagonizes the mobilization of  $Ca^{++}$  from intracellular stores (Malgodi and Chiou, 1974), was used. Pretreatment with TMB-8 dose-dependently antagonized the increase of pain threshold induced by ALCAR, suggesting that the release of  $Ca^{++}$  from intracellular stores induced by  $IP_3$  is necessary to produce ALCAR analgesia.

In addition to inducing  $IP_3$  formation, PLC causes the activation of PKC through stimulation of the production of DAG (Rebecchi and Pentyala, 2000). The involvement of PKC in the mechanisms that underlie the development of acute and persistent pain has been widely reported (Basbaum, 1999; Malmberg, 2000; Zimmermann, 2001; Kamei et al., 2001). In the present study, pretreatment with calphostin C and chelerytrine, selective, potent and membrane-permeable PKC inhibitors (Kobayashi et al., 1989; Herbert et al., 1990), dose-dependently enhanced the analgesia induced by ALCAR administration. Furthermore, activation of PKC by phorbol esters, such as PMA and PDBu (Nishizuka, 1992), antagonized the increase of pain threshold induced by ALCAR. These data indicate that activation of PKC constitutes a pathway involved in negative modulation of the central antinociceptive response induced by ALCAR.

The highest active doses of U-73122, U-73343, neomycin, LiCl, heparin, TMB-8, PMA and PDBu, as well as the inhibition of the expression of  $PLC\beta_1$ ,  $G_q$  and



G<sub>11</sub> by means of selective aODNs, in the absence of co-administration of ALCAR, did not reduced the licking latency values of mice in comparison with control groups. Similarly, calphostin C and chelerythrine, that potentiated ALCAR analgesia, when injected alone, did not increase mouse pain threshold. These results exclude not only that the prevention of ALCAR antinociception is due to hyperalgesic effect of the intracellular modulators used, but also that the action produced PKC blockers on ALCAR analgesia originates from antinociceptive properties of the pharmacological modulators employed.

The modulation of the intracellular events promoted by PLC activation can induce several side effects. It is widely known that the PKC activators PMA and PDBu are convulsant (Smith and Meldrum, 1992) and, similarly, LiCl can induce neurological toxicity characterized by tremors, convulsion, ataxia (Kores and Lader, 1997). All the compounds, at the highest active doses employed in the present study, did not cause any detectable modification in animals' gross behavior. At the same doses, all treatments did not impair motor coordination nor modify spontaneous motility nor inspection activity in comparison with control groups, excluding that the results obtained were due to animals' altered viability. It should be noted that higher doses of intracellular modulators could not be investigated since evident signs of toxicity, such as tremors, convulsions, etc. appeared. The induction of toxicity can also be considered as an indication not only of the diffusion of these compounds in the brain, but also of the consequent reaching of key targets by using the administration schedule employed in the present study.

Present data evidence the role of PLC-IP<sub>3</sub> pathway in the induction of ALCAR analgesia in a condition of acute thermal nociception in mice. Furthermore, the concomitant activation of PKC through DAG generation induced by PLC activation might represent a mechanism of negative modulation of ALCAR antinociception.

## Acknowledgements

This work was supported by grants from MIUR and Sigma-Tau Industrie Farmaceutiche Riunite S.p.A.

## References

- Ashkenazi, A., Winslow, J.W., Peralta, E.G., Peterson, G.L., Schimerlik, M.I., Capon, D.J., Ramachandran, J., 1987. An m2 muscarinic receptor subtype coupled to both adenylyl cyclase and phosphoinositide turnover. *Science* 238, 672–675.
- Bartolini, A., Ghelardini, C., Fantetti, L., Malcangio, M., Malmberg-Aiello, P., Giotti, A., 1992. Role of muscarinic receptor subtypes in central antinociception. *British Journal of Pharmacology* 105, 77–82.
- Basbaum, A.I., 1999. Distinct neurochemical features of acute and persistent pain. *Proceedings of the National Academy of Sciences of the United States of America* 96, 7739–7743.
- Bleasdale, J.E., Thakur, N.R., Gremban, R.S., Bundy, G.L., Fitzpatrick, F.A., Smith, R.J., Bunting, S., 1990. Selective inhibition of receptor-coupled phospholipase C-dependent processes in human platelets and polymorphonuclear neutrophils. *Journal of Pharmacology and Experimental Therapeutics* 255, 756–768.
- Caulfield, M.P., 1993. Muscarinic receptors—characterization, coupling and function. *Pharmacology and Therapy* 58, 319–379.
- Caulfield, M.P., Birdsall, N.J.M., 1998. International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. *Pharmacological Reviews* 50, 279–290.
- Chiechio, S., Caricasole, A., Barletta, E., Storto, M., Catania, M.V., Copani, A., Vertechy, M., Nicolai, R., Calvani, M., Melchiorri, D., Nicoletti, F., 2002. L-Acetylcarnitine induces analgesia by selective up-regulating mGlu2 metabotropic glutamate receptors. *Molecular Pharmacology* 61, 898–996.
- Felder, C.C., 1995. Muscarinic acetylcholine receptors: signal transduction through multiple effectors. *FASEB Journal* 9, 619–625.
- Ferris, C.D., Cameron, A.M., Haganir, R.L., Snyder, S.H., 1992. Quantal calcium release by purified reconstituted inositol 1,4,5-trisphosphate receptors. *Nature* 35, 350–352.
- Galeotti, N., Bartolini, A., Ghelardini, C., 2003. The phospholipase C-IP3 pathway is involved in muscarinic antinociception. *Neuropharmacology* 28, 888–897.
- Ghelardini, C., Galeotti, N., Gualtieri, F., Romanelli, M.N., Bartolini, A., 1996. S-(−)-ET126: a potent and selective M<sub>1</sub> antagonist in vivo and in vitro. *Life Sciences* 58, 991–1000.
- Ghelardini, C., Galeotti, N., Bartolini, A., 2000. Loss of muscarinic antinociception by antisense inhibition of M<sub>1</sub> receptors. *British Journal of Pharmacology* 129, 1633–1640.
- Ghelardini, C., Galeotti, N., Calvani, M., Mosconi, L., Nicolai, R., Bartolini, A., 2002. Acetyl-L-carnitine induces muscarinic antinociception in mice and rats. *Neuropharmacology* 43, 1180–1187.
- Haley, T.J., McCormick, W.G., 1957. Pharmacological effects produced by intracerebral injection of drugs in the conscious mouse. *British Journal of Pharmacology and Chemotherapy* 12, 12–15.
- Herbert, J.M., Augereau, J.M., Gleye, J., Maffrand, J.P., 1990. Chelerythrine is a potent and specific inhibitor of protein kinase C. *Biochemical and Biophysical Research Communications* 172, 993–999.
- Iwamoto, E.T., Marion, L., 1993. Characterization of the antinociception produced by intrathecally administered muscarinic agonists in rats. *Journal of Pharmacology and Experimental Therapeutics* 266, 329–338.
- Johnson, M.P., Kelly, G.M., Chamberlain, M., 2000. Blockade of pilocarpine-induced cerebellar phosphoinositide hydrolysis with metabotropic glutamate antagonists: evidence for an indirect control of granule cell glutamate release by muscarinic agonists. *Neuroscience Letters* 285, 71–75.
- Jonas, S., Sugimori, M., Llinas, R., 1997. Low molecular weight heparin a neuroprotectant? *Annals of the New York Academy of Sciences* 825, 389–393.
- Kamei, J., Mizoguchi, H., Narita, M., Tseng, L.F., 2001. Therapeutic potential of PKC inhibitors in painful diabetic neuropathy. *Expert Opinion on Investigational Drugs* 9, 1653–1664.
- Kennedy, E.D., Xhalliss, R.A., Rangan, C.I., Nahorski, S.R., 1990. Reduced inositol polyphosphate accumulation and inositol supply induced by lithium in stimulated cerebral cortex slices. *Biochemistry Journal* 267, 781–786.
- Kobayashi, E., Nakano, H., Morimoto, M., Tamaoki, T., 1989. Calphostin C (UCN-1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. *Biochemical and Biophysical Research Communications* 159, 548–553.
- Kores, B., Lader, M.H., 1997. Irreversible lithium neurotoxicity: an overview. *Clinical Neuropharmacology* 20, 283–299.

- Malagodi, M.H., Chiou, C.Y., 1974. Pharmacological evaluation of a new  $\text{Ca}^{++}$  antagonist, 8-(*N,N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8): studied in smooth muscle. *European Journal of Pharmacology* 27, 25–33.
- Malmberg, A.B., 2000. Protein kinase subtypes involved in injury-induced nociception. *Progress in Brain Research* 129, 51–59.
- Mignery, G.A., Sudhof, T.C., 1990. The ligand binding sites and transduction mechanism in the inositol-1,4,5-triphosphate receptor. *EMBO Journal* 9, 3893–3898.
- Naguib, M., Yaksh, T.L., 1997. Characterization of muscarinic receptor subtypes that mediate antinociception in the rat spinal cord. *Anesthesia and Analgesia* 85, 847–853.
- Nishizuka, Y., 1992. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* 258, 607–614.
- O'Callaghan, J.P., Holtzman, S.G., 1975. Quantification of the analgesic activity of narcotic antagonists by a modified hot-plate procedure. *Journal of Pharmacology and Experimental Therapeutics* 192, 497–505.
- Onofri, M., Fulgente, T., Melchionda, D., Marchionni, A., Tomasello, F., Salpietro, F.M., Alafaci, C., De Sanctis, E., Pennisi, G., Bella, R., Ventura, F., Morganti, A., 1995. L-acetyl-carnitine as a new therapeutic approach for peripheral neuropathies with pain. *International Journal of Clinical Pharmacology Research* 15, 9–15.
- Phiel, C.J., Klein, P.S., 2001. Molecular targets of lithium action. *Annual Reviews of Pharmacology and Toxicology* 41, 789–813.
- Phillippe, M., 1994. Neomycin inhibition of hormone-stimulated smooth muscle contractions in myometrial tissue. *Biochemical and Biophysical Research Communications* 205, 245–250.
- Quatraro, A., Roca, P., Donzella, C., Acampora, R., Marfella, R., Giugliano, D., 1995. Acetyl-L-carnitine for symptomatic neuropathy. *Diabetologia* 38, 123.
- Rebecchi, M.J., Pentyla, S.N., 2000. Structure, function and control of phosphoinositide-specific phospholipase C. *Physiological Reviews* 80, 1291–1335.
- Sanchez-Blazquez, P., Garzon, J., 1998. Delta opioid subtypes activates inositol-signaling pathways in the production of antinociception. *Journal of Pharmacology and Experimental Therapeutics* 285, 820–827.
- Scarpini, E., Sacilotto, G., Baron, P., Cusini, M., Scarlato, G., 1997. Effect of acetyl-L-carnitine in the treatment of painful peripheral neuropathies in HIV+ patients. *Journal of the Peripheral Nervous System* 2, 250–252.
- Smith, S.E., Meldrum, B.S., 1992. The protein kinase C activator-phorbol 12-myristate,13-acetate and 12,13-phorbol dibutyrate, are convulsant in the pico-nanomolar range in mice. *European Journal of Pharmacology* 213, 133–135.
- Smith, R.J., Sam, L.M., Justen, J.M., Bundy, G.L., Bala, G.A., Bleasdale, J.E., 1990. Receptor-coupled signal transduction in human polymorphonuclear neutrophils: effects of a novel inhibitor of phospholipase C-dependent processes on cell responsiveness. *Journal of Pharmacology and Experimental Therapeutics* 253, 688–697.
- Taylor, S.J., Chae, H.Z., Rhee, S.G., Exton, J.H., 1991. Activation of  $\beta 1$  isozyme of phospholipase C by  $\alpha$  subunits of the Gq class of G protein. *Nature* 350, 516–518.
- Vaught, J., Pelley, K., Costa, L.G., Sether, P., Enna, S.J., 1985. A comparison of the antinociceptive responses to GABA-receptor agonists THIP and baclofen. *Neuropharmacology* 24, 211–216.
- Wakdo, G.L., Northup, J., Perkins, J.P., Harden, T.K., 1983. Characterization of an altered membrane form of the beta-adrenergic receptor produced during agonist-induced desensitization. *Journal of Biological Chemistry* 258, 13900–13908.
- Yule, D.I., Williams, J.A., 1992. U73122 inhibits  $\text{Ca}^{2+}$  oscillations in response to cholecystokinin and carbachol but not to JMV-180 in rat pancreatic acinar cells. *Journal of Biological Chemistry* 267, 13830–13835.
- Zimmermann, M., 2001. Pathobiology of neuropathic pain. *European Journal of Pharmacology* 429, 23–37.